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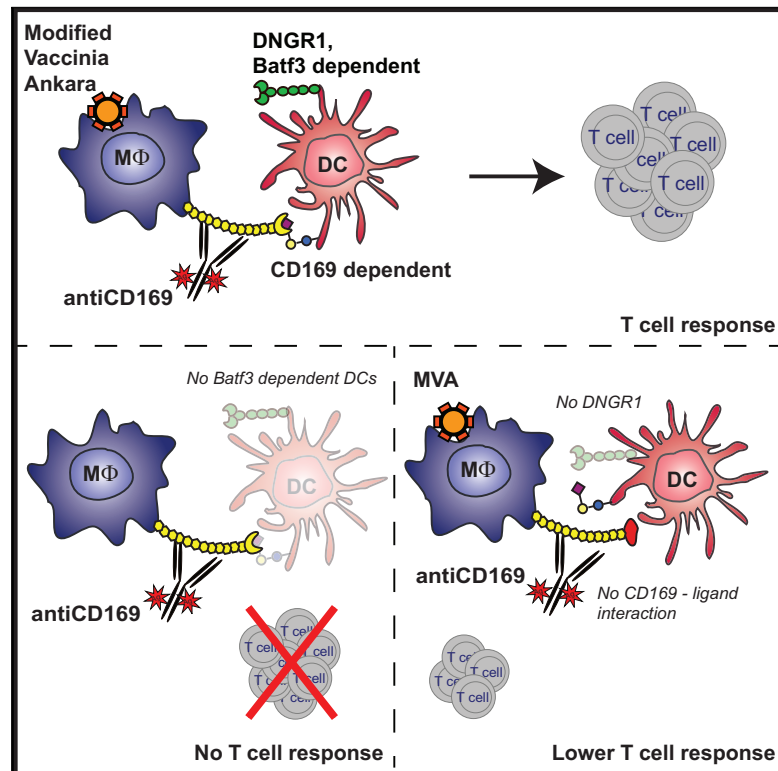
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## Functional CD169 on Macrophages Mediates Interaction with Dendritic Cells for CD8<sup>+</sup> T Cell Cross-Priming

### Graphical Abstract



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### In Brief

van Dinther et al. find that splenic CD169<sup>+</sup> macrophages transfer antigens to BATF3-dependent cDC1s in a CD169-dependent manner and that these cDC1s cross-prime CD8<sup>+</sup> T cell responses in a DNGR-1-dependent manner.

### Highlights

- CD169<sup>+</sup> macrophages rely on BATF3-dependent DCs to cross-prime CD8<sup>+</sup> T cells
- DNGR-1 on cDC1s improves cross-priming to antigens targeted to CD169<sup>+</sup> macrophages
- CD169 binds to sialic acids on CD8α<sup>+</sup> DCs
- CD169 binding promotes T cell priming after non-replicative vaccinia virus infection



# Functional CD169 on Macrophages Mediates Interaction with Dendritic Cells for CD8<sup>+</sup> T Cell Cross-Priming

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## SUMMARY

Splenic CD169<sup>+</sup> macrophages are located in the marginal zone to efficiently capture blood-borne pathogens. Here, we investigate the requirements for the induction of CD8<sup>+</sup> T cell responses by antigens (Ags) bound by CD169<sup>+</sup> macrophages. Upon Ag targeting to CD169<sup>+</sup> macrophages, we show that BATF3-dependent CD8 $\alpha$ <sup>+</sup> dendritic cells (DCs) are crucial for DNGR-1-mediated cross-priming of CD8<sup>+</sup> T cell responses. In addition, we demonstrate that CD169, a sialic acid binding lectin involved in cell-cell contact, preferentially binds to CD8 $\alpha$ <sup>+</sup> DCs and that Ag transfer to CD8 $\alpha$ <sup>+</sup> DCs and subsequent T cell activation is dependent on the sialic acid-binding capacity of CD169. Finally, functional CD169 mediates optimal CD8<sup>+</sup> T cell responses to modified vaccinia Ankara virus infection. Together, these data indicate that the collaboration of CD169<sup>+</sup> macrophages and CD8 $\alpha$ <sup>+</sup> DCs for the initiation of effective CD8<sup>+</sup> T cell responses is facilitated by binding of CD169 to sialic acid containing ligands on CD8 $\alpha$ <sup>+</sup> DCs.

## INTRODUCTION

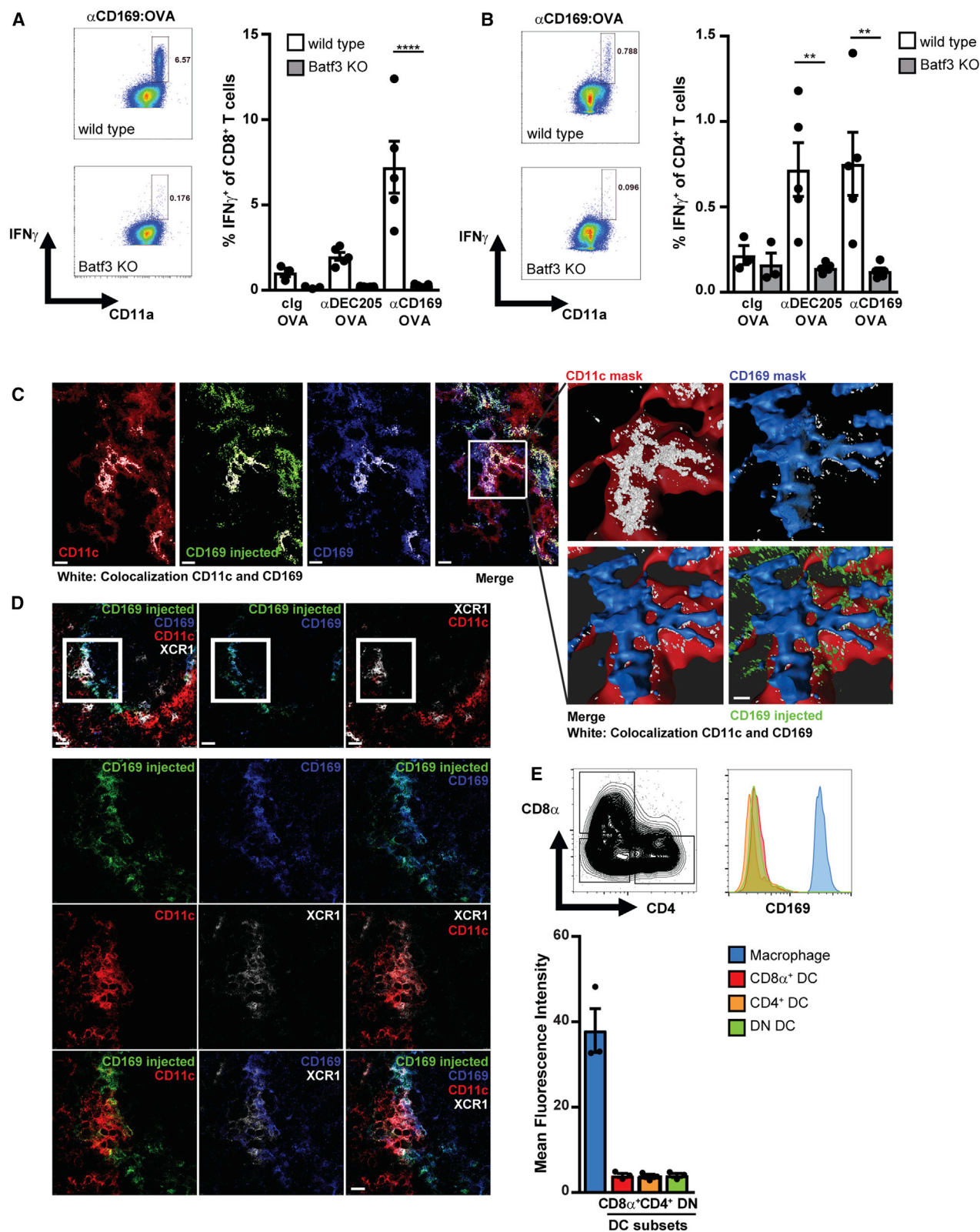
While lymph nodes receive antigen (Ag) via the afferent lymph vessels, the spleen filters the blood in the marginal zone and red pulp. Macrophages characterized by high expression of CD169, also known as Sialoadhesin or sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1), are situated at both sites of Ag entry, the subcapsular sinus and marginal sinus (Martinez-Pomares and Gordon, 2012). An intact layer of CD169<sup>+</sup> macrophages is necessary for removal of pathogens and prevention of further spreading of infection (Gupta et al., 2016; Iannacone

et al., 2010; Junt et al., 2007; Seiler et al., 1997) and they rapidly stimulate several innate lymphoid cell types, thereby promoting an early wave of innate host defense (Barral et al., 2010; Kastenmüller et al., 2012; Kawasaki et al., 2013).

CD169<sup>+</sup> macrophages are situated on top of B cell follicles, take up Ag such as immune complexes and viruses, present these Ags in intact form to follicular B cells, and induce germinal center B cell responses (Berney et al., 1999; Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007, 2009; Veninga et al., 2015). However, the role of CD169<sup>+</sup> macrophages in the priming of CD8<sup>+</sup> T cell responses is debated (Asano et al., 2011; Backer et al., 2010; Bernhard et al., 2015).

CD169<sup>+</sup> macrophages are frequently the primary cell type infected during a viral infection (Eloranta and Alm, 1999; Iannacone et al., 2010) and while the CD169 molecule itself acts as a viral receptor (Delputte et al., 2007; Erikson et al., 2015; Puryear et al., 2013; Sewald et al., 2015), CD169<sup>+</sup> macrophages are generally considered dispensable for the initiation of T cell responses (Aichele et al., 2003; Winkelmann et al., 2014). CD169<sup>+</sup> macrophages have been shown to allow viral replication, which results in high amounts of viral Ags and amplification of T and B cell responses (Honke et al., 2011). This suggests that pathogen uptake and Ag generation are the main functions of CD169<sup>+</sup> macrophages, while dendritic cells (DCs) are essential for CD8<sup>+</sup> T cell priming (den Haan et al., 2000; Jung et al., 2002; Segura and Amigorena, 2015). In our previous studies, we observed Ag presentation to CD8<sup>+</sup> T cells by cross-presenting CD8 $\alpha$ <sup>+</sup> DCs (classical DC1, cDC1) when Ag was targeted to CD169<sup>+</sup> macrophages (Backer et al., 2010). Recently, Bernhard et al. (2015) showed that after adenoviral infection of CD169<sup>+</sup> macrophages, long-lived Ag was cross-presented by CD8 $\alpha$ <sup>+</sup> DCs to CD8<sup>+</sup> T cells. Together, these studies indicate a functional collaboration between CD169<sup>+</sup> macrophages and cross-presenting DCs in the activation of CD8<sup>+</sup> T cells and imply an interaction between the two cell types to enable Ag transfer. However, the molecular interactions involved in this process are completely unknown.





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One of the molecules indicated to play a role in cellular communication is CD169 itself. CD169 is a lectin receptor that binds sialylated glycoproteins and glycolipids on both endogenous cells as well as pathogens (Crocker and Gordon, 1989; Crocker et al., 1994; Macauley et al., 2014). In previous studies, CD169 has been shown to mediate binding to neutrophils and to innate-like lymphocytes (Crocker et al., 1995; van den Berg et al., 1992; Zhang et al., 2016). In the present study, we find that, when Ags are specifically targeted to CD169<sup>+</sup> macrophages, presentation by BATF3-dependent cross-presenting DCs is essential for cross-priming of CD8<sup>+</sup> T cells. Our studies demonstrate that CD169 is involved in Ag transfer to CD8 $\alpha$ <sup>+</sup> DCs and priming of CD8<sup>+</sup> T cells. Finally, the activation of non-replicative vaccinia virus-specific CD8<sup>+</sup> T cells is hampered when the binding activity of CD169 was blocked. These results indicate that CD169<sup>+</sup> macrophages interact with DC1s in a CD169-dependent manner and that this interaction enhances BATF3-dependent, DNNGR-1-mediated cross-priming of CD8<sup>+</sup> T cells.

## RESULTS

### T Cell Responses after Ag Targeting to CD169<sup>+</sup> Macrophages Rely on BATF3-Dependent DCs

Previously, we showed that Ag targeting to CD169<sup>+</sup> macrophages resulted in Ag presentation by CD8 $\alpha$ <sup>+</sup> DCs to CD8<sup>+</sup> T cells (Backer et al., 2010). To investigate the requirement of cross-presenting DCs to present Ags bound by CD169<sup>+</sup> macrophages, we used BATF3-deficient mice, which contain significantly decreased numbers of cross-presenting CD8 $\alpha$ <sup>+</sup> DCs, but do contain normal frequencies of CD169<sup>+</sup> macrophages (Figures S1A and S1B) (Hildner et al., 2008). We immunized wild-type (WT) and BATF3-deficient mice with antibody (Ab)-ovalbumin (OVA) conjugates targeted to DEC205<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs or CD169<sup>+</sup> macrophages in the presence of adjuvants. Both OVA targeting to DEC205 or CD169 induced OVA-specific effector and memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells in a BATF3-dependent manner (Figures 1A, 1B, and S1C). These results demonstrate that CD169<sup>+</sup> macrophages do not cross-present exogenous Ags and that T cell cross-priming relies on cross-presenting DCs.

Because Ag transfer between CD169<sup>+</sup> macrophages and DCs would imply physical interactions between these cells, we determined the localization of DCs and CD169<sup>+</sup> macrophages in mice injected with Alexa 488-labeled anti-CD169 ( $\alpha$ CD169-488)

Ab plus adjuvant by fluorescence microscopy. DCs and CD169<sup>+</sup> macrophages were located in close contact with each other, and the injected Ab was observed on contact points between the macrophages and the DCs (Figure 1C). Staining for XCR1, which is a unique marker for BATF3-dependent DCs, demonstrated multiple interactions between XCR1<sup>+</sup> cDC1s with CD169<sup>+</sup> macrophages carrying CD169-488 (Figure 1D). Together, these results indicate colocalization between CD169<sup>+</sup> macrophages and cross-presenting DCs, and we hypothesize that this interaction would enable Ag transfer.

We and others have observed that CD8 $\alpha$ <sup>+</sup> DCs show some CD169 staining after isolation using enzymatic digestion (Figure S1D) (Segura et al., 2010). If CD8 $\alpha$ <sup>+</sup> DCs would express CD169 molecules, then direct targeting to DCs would be possible. However, we and others did not detect CD169 RNA expression in sorted CD8 $\alpha$ <sup>+</sup> DCs (Figure S1E) (GEO: GSE6259) (Dudziak et al., 2007). The presence of CD169 on other cell types can be caused by the isolation procedure (Zhang et al., 2016). To exclude this phenomenon, we used multiplex confocal microscopy, 3D image surface rendering, and subsequent histocytometry analysis of murine spleens to quantify CD169 expression *ex vivo* on APC subsets. This analysis showed no expression of CD169 by DC subsets in spleen sections (Figure 1E). Together, these results suggest that CD8 $\alpha$ <sup>+</sup> DCs do not express CD169, but do interact with CD169<sup>+</sup> macrophages and are essential for the activation of CD8<sup>+</sup> T cell responses.

### Ags Bound by CD169<sup>+</sup> Macrophages Are Transferred to CD8 $\alpha$ <sup>+</sup> DCs

To investigate Ag transfer between CD169<sup>+</sup> macrophages and DCs, we analyzed the transfer of  $\alpha$ CD169-488 to DCs in the presence of adjuvant by flow cytometry. At 10 min after injection, half of the macrophages were labeled with fluorescent Ab (Figures S2A and S2B), while this was negligible for CD8 $\alpha$ <sup>+</sup>, CD4<sup>+</sup>, or double negative (DN) DCs (Figures 2A–2C). Two hours after immunization, 75% of the macrophages (Figures S2A and S2B) and 15%–20% of CD8 $\alpha$ <sup>+</sup> DCs and DN DCs, and to a lower extent CD4<sup>+</sup> DCs, were labeled (Figures 2A–2C). The increase in mean fluorescence was highest in CD8<sup>+</sup> DCs at different time points after injection (Figure 2D). Analysis of transferred  $\alpha$ CD169-488 in DC subsets in BATF3-deficient mice revealed an increase in Ag transfer to CD4<sup>+</sup> DCs (Figures 2E–2H). These results suggest a preferential interaction between CD169<sup>+</sup> macrophages with CD8 $\alpha$ <sup>+</sup> DCs.

### Figure 1. T Cell Activation after Ag Targeting to CD169<sup>+</sup> Macrophages Requires BATF3-Dependent DCs

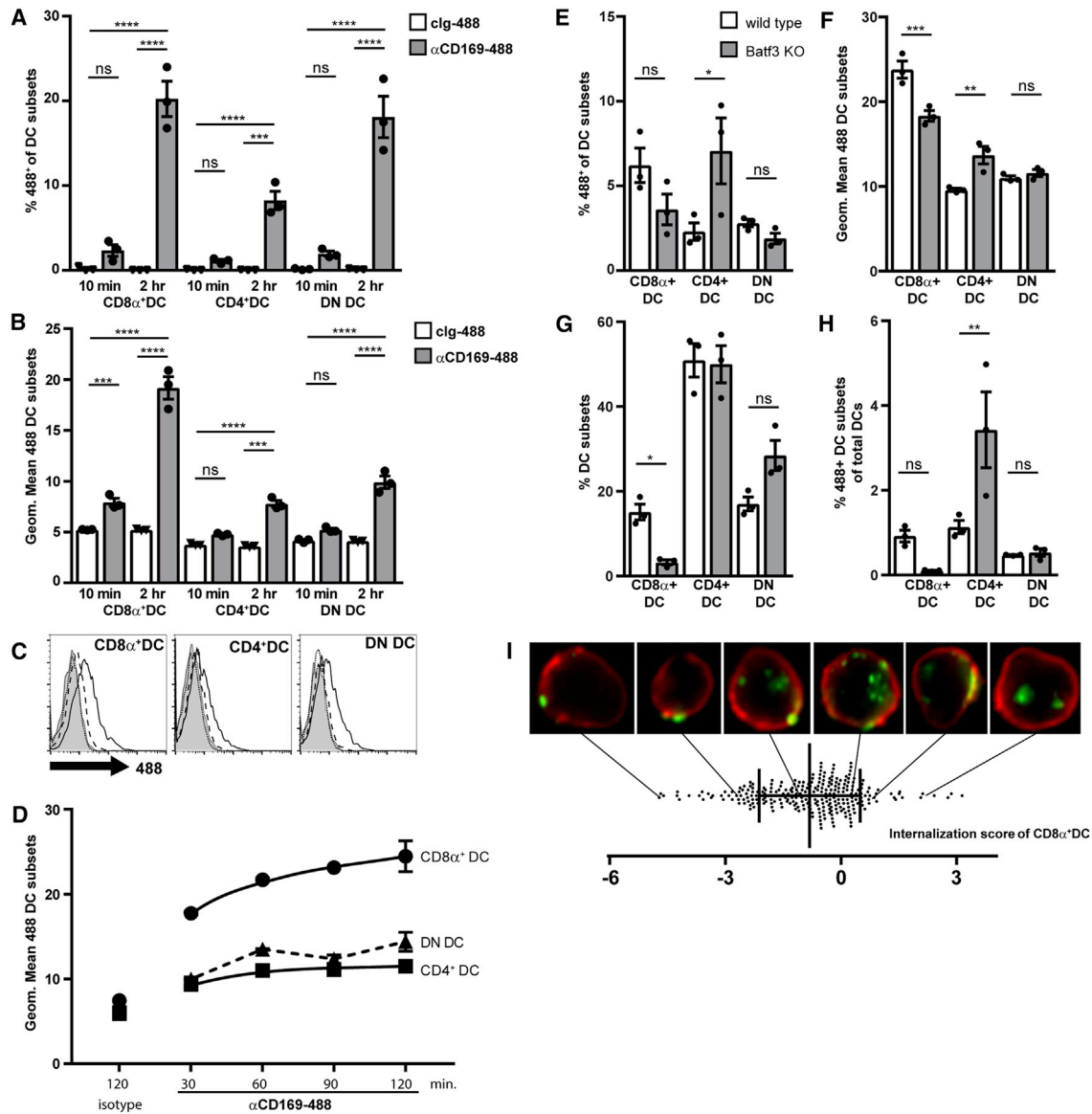
(A and B) Percentage of OVA-specific IFN $\gamma$  producing CD8<sup>+</sup> (A) and CD4<sup>+</sup> (B) CD11a<sup>+</sup> T cells after *in vitro* re-stimulation with H-2K<sup>b</sup>-restricted OVA<sub>257–264</sub> peptide or I-A<sup>b</sup>-restricted OVA<sub>262–276</sub> peptide, in C57BL/6 (WT, wild-type) and BATF3-deficient knockout (KO) mice 9 days after immunization (1  $\mu$ g of indicated Ab-OVA with 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C)). Representative flow cytometry plots and mean  $\pm$  SEM of one representative experiment of two experiments using 3–5 mice/group. Statistical analysis one-way ANOVA with Bonferroni's multiple comparison test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; n.s., non-significant.

(C) CD11c (red), injected anti-CD169 (green), CD169 (blue), and in white a co-localization mask of CD11c and CD169 (merge without co-localization mask). Zoom of the indicated area with cell masks for CD11c (red) and CD169 (blue), co-localization mask (white), and injected anti-CD169 (green). Scale bars, 5  $\mu$ m and 3  $\mu$ m in the zoom.

(D) Representative image of CD169<sup>+</sup> macrophage interacting with XCR1<sup>+</sup> DC. CD11c (red), injected anti-CD169 (green), CD169 (blue), and XCR1 (white). Scale bars, 25  $\mu$ m and 10  $\mu$ m in the zoom.

(E) *In situ* CD169 expression on splenic macrophages and DC subsets based on multiplex confocal microscopy and 3D image surface rendering of whole spleen sections (n = 3).

See also Figure S1F.



**Figure 2. Ags Targeted to CD169<sup>+</sup> Macrophages Are Transferred to DCs**

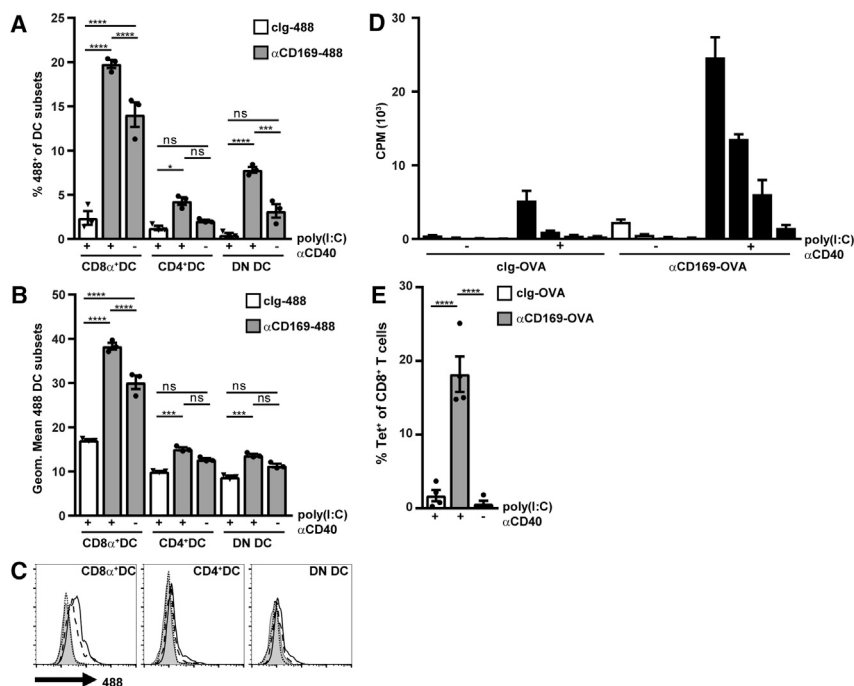
(A–D) Wild-type mice were injected with 1  $\mu$ g of the indicated Ab-Alexa488 with 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). (A) Percentage of Alexa488<sup>+</sup> DCs, (B) geometric mean of Alexa488 signal per DC subset, and (C) representative histogram of each DC subset at 10 min (dotted line) and 2 hr (solid line) after immunization (gray line isotype control), one representative experiment of two experiments each with 3 mice/group. (D) Geometric mean  $\pm$  SEM of Alexa488 signal per DC subset on indicated time points after injection. 3 mice/group.

(E–H) Wild-type mice or BATF3-KO mice were injected with 1  $\mu$ g of the indicated Ab-Alexa488 with 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). (E) Percentage of Alexa488<sup>+</sup> DCs and (F) geometric mean of Alexa488 signal per DC subset. (G) percentage of DC subsets per group. (H) 488<sup>+</sup> cells per subset as a percentage of total DCs. Mean  $\pm$  SEM of one representative experiment of two with 3 mice per group.

(I) Representative images of isolated CD8 $\alpha$ <sup>+</sup> DCs containing Alexa488 signal 2 hr after Ag targeting. 3 mice per group were pooled, representative of 4 experiments. Negative internalization scores indicate that the majority of Alexa488 signal is membrane-associated, while positive internalization scores indicate intracellular presence of Alexa488 signal. Statistical analysis one-way ANOVA with Bonferroni's multiple comparison test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; n.s., non-significant.

Using imaging flow cytometry, CD8 $\alpha$ <sup>+</sup> DCs were shown to carry both membrane-associated and intracellular fluorescent content, indicating uptake of  $\alpha$ CD169-488 (Figure 2I). The fluorescence was concentrated in round structures both at the cell membrane as well as intracellular, which is in line with the

observed binding and uptake of cellular vesicles during the 2 hr after immunization. The combined results of these studies indicate that Ags are transferred from CD169<sup>+</sup> macrophages to DCs, and the transfer appears to be more efficient to CD8 $\alpha$ <sup>+</sup> and to DN DCs than to CD4<sup>+</sup> DCs.



**Figure 3. Adjuvant Enhances Antigen Transfer from Macrophages to DCs**

(A–C) Wild-type mice were injected with 1  $\mu$ g of the indicated Ab-Alexa488 in the presence or absence of 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). (A) Percentage of Alexa488<sup>+</sup> DC subsets and (B) geometric mean of Alexa488 signal per DC subset 2 hr after injection using 3 mice/group. (C) Representative histograms of each DC subset at 2 hr after injection of Ab-Alexa488 with adjuvant (solid line) or without adjuvant (dotted line), gray area for the isotype control. Mean  $\pm$  SEM of one representative of 2 experiments is shown.

(D) Wild-type mice were injected with 1  $\mu$ g of indicated Ab-OVA in the presence or absence of 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). 16 hr later DCs were used as stimulators for an OT-I proliferation assay, measured by <sup>3</sup>H-Thymidine incorporation. Graph shows mean counts per minute (CPM)  $\pm$  SEM of triplicate co-cultures of 10<sup>5</sup> OT-I T cells together with a 3-fold 3-step titration of DCs starting at 2.5  $\times$  10<sup>5</sup> DCs. One representative experiment of 3 is shown. (E) Percentage of H-2K<sup>b</sup>-SIINFEKL-tetramer<sup>+</sup> CD8<sup>+</sup> T cells 7 days after immunization. Mean  $\pm$  SEM of 3 mice per group. Statistical analysis one-way ANOVA with Bonferroni's multiple comparison test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; n.s., non-significant.

### Adjuvant Enhances Ag Transfer from CD169<sup>+</sup> Macrophages to CD8 $\alpha$ <sup>+</sup> DCs

Because all previous experiments were performed in the presence of adjuvant, we questioned whether Ag transfer was enhanced by the presence of adjuvant. We detected a decrease in the percentage and intensity of fluorescent CD8 $\alpha$ <sup>+</sup> and DN DCs in mice injected with  $\alpha$ CD169-488 without adjuvant, although still a significant amount of Ag transfer remained (Figures 3A–3C). To test the functional relevance of Ag transfer in the absence of adjuvant, we used DCs from mice injected with  $\alpha$ CD169-OVA without adjuvant to stimulate OT-I T cells *in vitro*. These DCs provided minimal stimulation for OT-I T cell activation, even though they could stimulate T cell responses after exogenous peptide loading, indicating that the DCs did not have any intrinsic defect to stimulate T cells (Figures 3D and S3A). In addition, immunization with  $\alpha$ CD169-OVA conjugate in the absence of adjuvant did not lead to OVA-specific T cell responses *in vivo* (Figure 3E). Our results demonstrate that adjuvant enhances Ag transfer from CD169<sup>+</sup> macrophages to DCs and is absolutely essential in the activation of T cell responses.

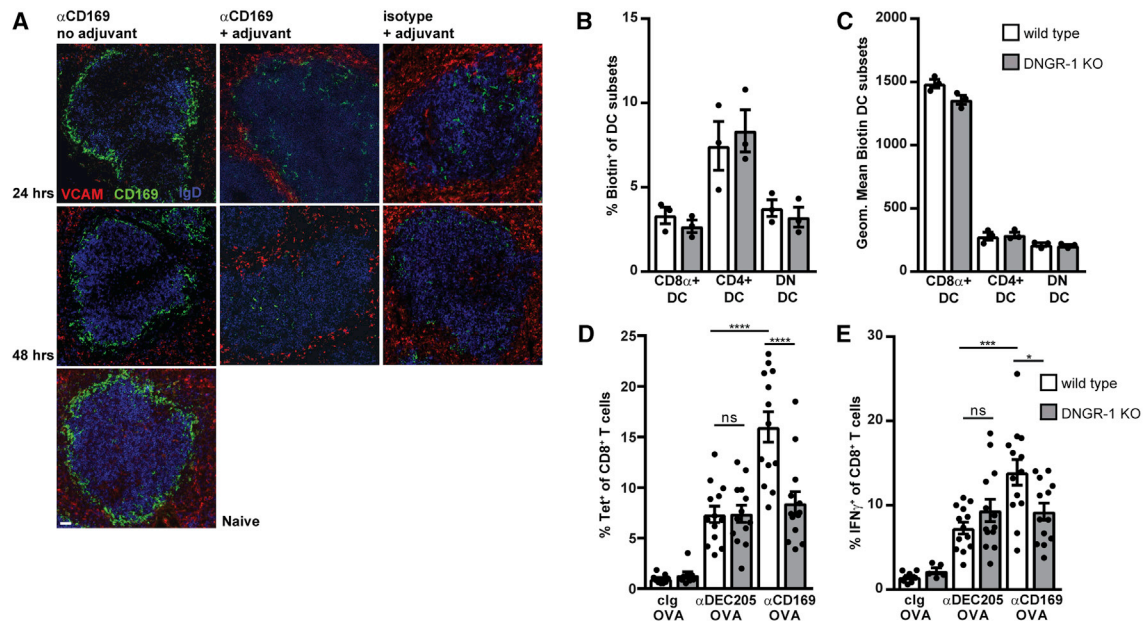
### DNDR-1 Expression on Cross-Presenting DCs Enhances Cross-Priming of CD8<sup>+</sup> T Cell Responses

A well-known feature of CD8 $\alpha$ <sup>+</sup> DCs is that they can efficiently recognize and cross-present cell-associated Ags from dying cells (den Haan et al., 2000; Iyoda et al., 2002; Schulz and Reis e Sousa, 2002). Recently, lymph node CD169<sup>+</sup> macrophages were shown to die and disappear after infection or inflammation (Gaya et al., 2015; Sagoo et al., 2016). Consistent with this, we detected a significant decrease in CD169<sup>+</sup> macrophages and migration of the remaining CD169<sup>+</sup> macrophages into the B cell follicles 24 and 48 hr after adjuvant injection, while

$\alpha$ CD169-OVA conjugate in the absence of adjuvant did not induce this disappearance (Figure 4A). Because macrophages also quickly die following isolation (Zhang et al., 2016) (Figure S4A), we hypothesized that they might die upon activation *in vivo* and that this process would enhance recognition by CD8 $\alpha$ <sup>+</sup> DNDR-1-expressing DCs. DNDR-1 is a C-type lectin receptor that recognizes F-actin on dead cells and stimulates cross-presentation of dead-cell-derived material by CD8 $\alpha$ <sup>+</sup> DCs and the activation of CD8<sup>+</sup> T cell responses in viral infections (Ahrens et al., 2012; Iborra et al., 2012, 2016; Sancho et al., 2009). Normal numbers of both CD169<sup>+</sup> macrophages and DC were detected in DNDR-1-deficient (Clec9a<sup>gfp/gfp</sup>) mice (Figure S4B). While Ag transfer from CD169<sup>+</sup> macrophages to DCs was unaffected in DNDR-1-deficient mice (Figures 4B and 4C), CD8<sup>+</sup> T cell responses were significantly lower in DNDR-1-deficient mice following immunization (Figures 4D and 4E). These results are in line with previous studies that demonstrated that DNDR-1 recognition of F-actin is not essential for uptake, but enhances cross-presentation of cell-associated Ag to T cells (Sancho et al., 2009; Zelenay et al., 2012). In conclusion, our data indicate that adjuvants play a relatively minor role in Ag transfer, but are essential for the activation of CD8<sup>+</sup> T cell responses and may stimulate DNDR-1-mediated cross-priming of T cells by CD8 $\alpha$ <sup>+</sup> DC.

### CD169 Binds to Sialic Acids on CD8 $\alpha$ <sup>+</sup> DCs, Promotes Ag Transfer, and T Cell Cross-Priming

The CD169 molecule is a sialic acid binding lectin with a preference for  $\alpha$ 2,3-linked sialic acids that is involved in cellular interactions (Crocker et al., 1995; van den Berg et al., 1992). We hypothesized a role for CD169 in DC interaction and Ag transfer and stained splenocytes with plant lectins that have a



specificity for  $\alpha$ 2,3 sialic acids (MAL-II) and  $\alpha$ 2,6 sialic acids (SNA) (Figure 5A). DCs showed highest expression of  $\alpha$ 2,3 sialic acids compared to B cells and neutrophils, and low expression of  $\alpha$ 2,6 sialic acids. Recombinant CD169Fc proteins demonstrated more binding to DCs than to neutrophils (Figure 5B). Especially CD8 $\alpha$ <sup>+</sup> and to a lesser extent CD4<sup>+</sup> and DN DCs bound MAL-II (Figure 5C) and recombinant CD169Fc (Figure 5D). Binding was dependent on sialic acids as neuraminidase treatment of DCs abrogated the interaction (Figure 5E) and recombinant CD169Fc containing an alanine substitution in the sialic acid binding domain (R97A) that abolishes sialic acid binding, did not bind to DCs or to other splenocytes (Figures 5B and 5D) (Vinson et al., 1996). These data indicate that CD169 on macrophages can bind to sialic acids on DCs and this may facilitate the interaction between macrophages and DCs necessary for the transfer of CD169<sup>+</sup> macrophage-derived Ag to cross-presenting CD8 $\alpha$ <sup>+</sup> DCs.

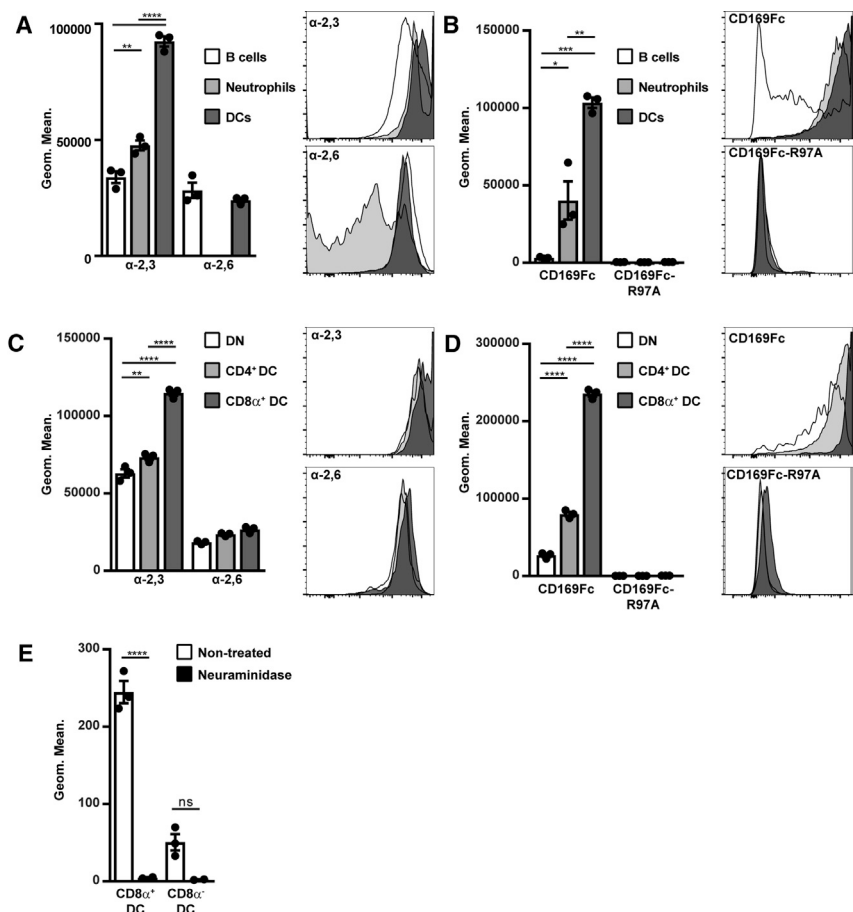
To investigate the role of CD169-mediated recognition of sialic acids in the transfer of Ag and the activation of T cells, we injected Sn-KI mice expressing a mutated CD169, unable to bind sialic acids because of the amino acid substitutions R97A and W2Q, with  $\alpha$ CD169-488 and measured the transfer of  $\alpha$ CD169-488 to DCs 2 hr after injection (Klaas et al., 2012). Sn-KI mice contain normal numbers of the DC subsets and CD169-expressing macrophages (Figures S5A–S5C). Both the

percentage of Alexa488<sup>+</sup> DCs and the MFI on the DC subsets were decreased by 50% or more in Sn-KI mice (Figures 6A and 6B). This indicates that Ag transfer between CD169<sup>+</sup> macrophages and DCs depends on the presence of functional sialic-acid binding CD169 on the macrophages. Accordingly, CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses following immunization with  $\alpha$ CD169-OVA conjugates were reduced in Sn-KI mice compared to wild-type mice, whereas immunization with  $\alpha$ DEC205-OVA conjugates was not affected (Figures 6C and 6D). These experiments show that CD169 binds to CD8 $\alpha$ <sup>+</sup> DCs in a sialic acid-dependent manner, and functional CD169 enhances Ag transfer and contributes to T cell cross-priming *in vivo*.

#### Uptake of Soluble Ag and Immune Complexes by DCs Is Not Dependent on Functional CD169

To determine whether functional CD169 is important for other types of Ag, we investigated the uptake of soluble antigen and immune complexes by DC subsets in wild-type and Sn-KI mice. Two hours after injection of Alexa 488-labeled OVA, a similar percentage of all DC subsets was Alexa488-positive and this was not changed in Sn-KI mice (Figures 6E, 6F, and S6A). Similarly, the uptake of immune complexes of OVA-488 rabbit anti-OVA Ab was present in all DC subsets and not affected by a non-functional CD169 molecule (Figures 6G, 6H,





**Figure 5. CD169 Binds to CD8 $\alpha$ <sup>+</sup> DCs in a Sialic Acid-Dependent Manner**

(A–D) Binding of MAL-II and SNA lectins ( $\alpha$ 2,3 and  $\alpha$ 2,6-sialic acid specific) and recombinant CD169-Fc and CD169-R97A mutant to B220<sup>+</sup> B cells, Gr1<sup>+</sup> CD11b<sup>+</sup> neutrophils, and CD11c<sup>+</sup>MHC class II<sup>+</sup> DCs (A) and (B) and to CD8<sup>+</sup> and CD8<sup>−</sup> CD11c<sup>+</sup> MHC class II<sup>+</sup> DCs (C) and (D).

(E) Binding of CD169-Fc to neuraminidase-treated and non-treated DCs.

Geometric mean  $\pm$  SEM of 3 mice/group and one representative experiment from at least two independent experiments is shown. Statistical analysis one-way ANOVA with Bonferroni's multiple comparison test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; n.s., non-significant.

T cell help via BATF3-dependent DCs (Eickhoff et al., 2015), while the numbers of KLRG-1<sup>high</sup> cells were not affected (Figure 7E). Together, our results indicate that in MVA vaccinia virus infection functional CD169 is necessary to obtain optimal cross-presentation and induction of CD8<sup>+</sup> T cell responses.

In contrast to T cell responses after MVA infection, T cell responses after intravenous vesicular stomatitis virus infection were not influenced by blocking CD169-binding to its ligands (Figure S6C). In conclusion, our analysis of two different viral models suggest that CD169-mediated interaction between CD169<sup>+</sup> macrophages

and S6B). These results suggest that DCs directly take up soluble Ag and immune complexes independently of CD169-mediated interaction with macrophages.

### Blocking CD169 Interactions Impairs T Cell Responses after Modified Vaccinia Ankara Virus Infection

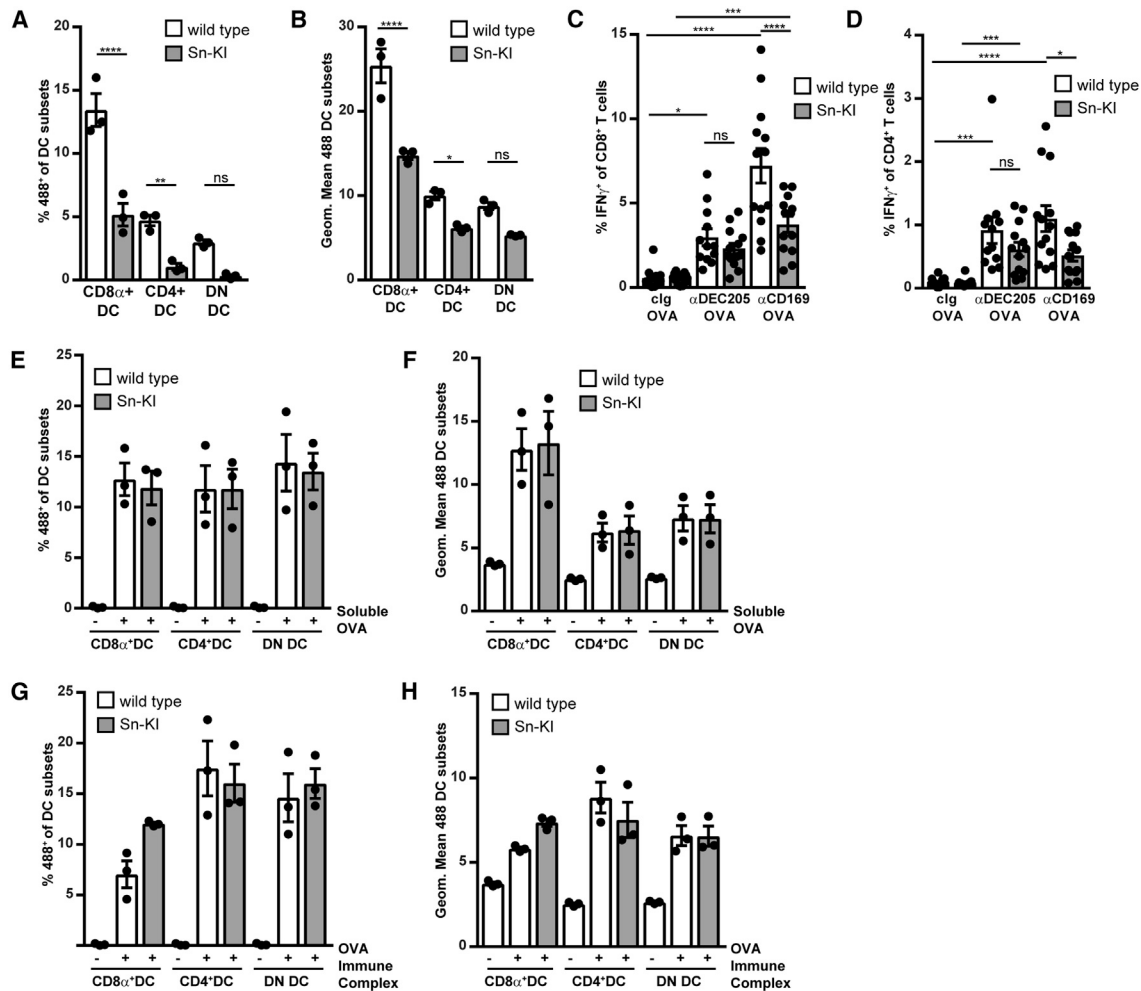
CD8<sup>+</sup> T cell responses induced by modified vaccinia Ankara (MVA) virus infection are dependent on BATF3-dependent cross-presenting DCs and DNGR-1-mediated cross-priming (Iborra et al., 2012, 2016). MVA infects CD169<sup>+</sup> macrophages in lymph nodes and induces cell death (Sagoo et al., 2016). We infected mice with MVA virus in the footpad and analyzed the effect of CD169-blocking antibodies on the MVA-specific CD8<sup>+</sup> T cell response in the draining lymph node and spleen 7 days after infection. The CD169-blocking Abs did not affect CD169<sup>+</sup> macrophage presence in spleen and lymph node (Figure S6B). We observed significantly decreased T cell responses specific for the B8R immunodominant peptide from MVA in both lymph nodes as well as spleen as detected by tetramer staining and intracellular cytokine staining after *in vitro* restimulation, which indicates that functional CD169 is necessary to mediate cross-priming of MVA-specific CD8<sup>+</sup> T cells (Figures 7A–7C). Moreover, we found a significant decrease in KLRG-1<sup>low</sup> cells when CD169 was blocked (Figure 7D), fitting with a decrease in Ag cross-presentation and limited CD4<sup>+</sup>

and CD8 $\alpha$ <sup>+</sup> DCs contributes to the generation of CD8<sup>+</sup> T cell immunity in those viral infections in which cross-presentation is essential.

### DISCUSSION

CD169<sup>+</sup> macrophages in lymphoid organs are the first cells to bind incoming pathogens and act as gatekeepers to prevent further dissemination of pathogens. While their excellent capacity to present Ags to B cells is well established, their specific role in the activation of T cell responses and the molecular mechanisms of their interaction with cross-presenting DCs are still not known. Our studies demonstrate unequivocally that BATF3-dependent DCs and CD169<sup>+</sup> macrophages have a CD169-dependent interaction that facilitates Ag transfer and DNGR1-mediated cross-presentation and thereby enables the efficient induction of CD8<sup>+</sup> T cell responses to viruses that depend on cross-presentation.

Interestingly, a recent study on human DCs describes a division of labor between DC subsets in which Ag acquisition and Ag presentation functions are separated over different DC subsets (Silvin et al., 2017). We propose a similar division of labor for CD169<sup>+</sup> macrophages and BATF3-dependent cross-presenting DCs in the mouse. Multiple studies have illustrated that CD169<sup>+</sup> macrophages bind and are infected by different

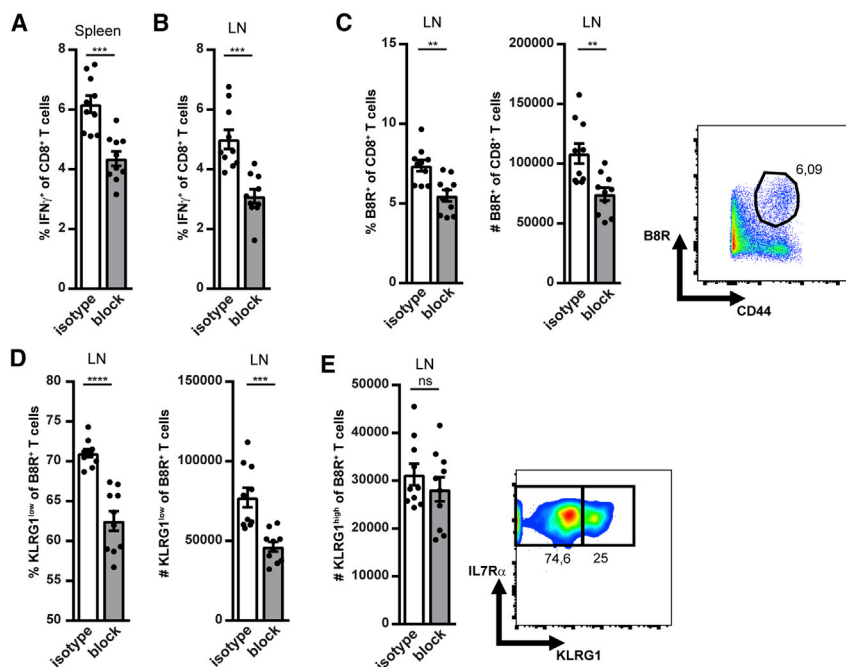


**Figure 6. CD169 Promotes Ag Transfer and T Cell Cross-Priming**

(A and B) Wild-type or Sn-KI mice were injected with 1  $\mu$ g of the indicated Ab-488 with 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). (A) Percentage of Alexa488<sup>+</sup> DC subsets and (B) geometric mean of Alexa488 signal per DC subset two hours after injection using 3 mice/group. Mean  $\pm$  SEM of one representative of 2 experiments is shown. (C and D) Percentage of OVA-specific IFN- $\gamma$ -producing CD8<sup>+</sup> (C) and CD4<sup>+</sup> (D) CD11a<sup>+</sup> T cells after *in vitro* re-stimulation with H-2K<sup>b</sup>-restricted OVA<sub>257–264</sub> peptide or I-A<sup>b</sup>-restricted OVA<sub>262–276</sub> peptide, respectively, in spleens of wild-type and Sn-KI mice 9 days after immunization with 1  $\mu$ g of indicated Ab-OVA with 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). Graphs show the combined mean  $\pm$  SEM from 3 experiments of total 13 mice/group. (E and F) Wild-type or Sn-KI mice were injected with 100  $\mu$ g rabbit-anti-OVA Ab and 30 min later with 5  $\mu$ g soluble Alexa488-labeled OVA, resulting in the formation of immune complexes. (G and H) Wild-type or Sn-KI mice were injected with 50  $\mu$ g soluble Alexa488-labeled OVA. (E–H) Percentage of Alexa488<sup>+</sup> DC subsets (E and G) and geometric mean of Alexa488 signal per DC subset (F and H) 2 hr after injection using 3 mice/group. Mean  $\pm$  SEM of one representative of two experiments is shown. Statistical analysis one-way ANOVA with Bonferroni's multiple comparison test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; n.s., non-significant.

types of viruses (Bernhard et al., 2015; Honke et al., 2011; Junt et al., 2007; Sagoo et al., 2016; Sewald et al., 2015). In the case of VSV infection, this leads to enhanced local restricted replication of the virus and the production of Ag that enhances B and T cell responses (Honke et al., 2011). Vaccinia virus infection of CD169<sup>+</sup> cells has been shown to lead to inflammasome activation and cell death (Sagoo et al., 2016). Bacterial infection and inflammatory agents induced disappearance of subcapsular sinus CD169<sup>+</sup> macrophages in the lymph node (Gaya et al., 2015). Similarly, we observed the disappearance of splenic marginal zone CD169<sup>+</sup> macrophages after injection with adjuvant.

Although we formally did not show CD169<sup>+</sup> macrophage cell death, we hypothesized that adjuvant-induced macrophage cell death would lead to generation of ligands for DNCR-1 expressed on Batf-3-dependent DCs that would enable cross-presentation of cell-associated antigens (Ahrens et al., 2012; Iborra et al., 2012; Sancho et al., 2009; Zelenay et al., 2012). Indeed, experiments in DNCR-1-deficient mice demonstrated a role for DNCR-1 in the cross-priming of T cell responses after Ag targeting to CD169<sup>+</sup> macrophages, although we did not observe a major role for adjuvant or DNCR-1 for Ag transfer to DCs as has been previously reported (Iborra et al., 2012).



**Figure 7. In Vivo Blockade of CD169 Impairs the Activation of MVA-Specific CD8<sup>+</sup> T Cell Responses**

(A–D) Percentage of MVA-specific IFN $\gamma$ -producing CD8<sup>+</sup> T cells in spleen (A) and LNs (B) after *in vitro* restimulation with MVA-derived B8R<sup>+</sup> peptide 7 days after footpad infection with  $5 \times 10^5$  PFU MVA in the presence of CD169-blocking Abs or isotype control. Percentage and number of B8R<sup>+</sup> tetramer-specific CD8<sup>+</sup> T cells (C) and of KLRG1<sup>low</sup> B8R<sup>+</sup> CD8<sup>+</sup> T cells (D) in LNs from mice 7 days after footpad infection. (E) Number of KLRG1<sup>high</sup> B8R<sup>+</sup> CD8<sup>+</sup> T cells in LNs from mice 7 days after footpad infection with  $5 \times 10^5$  PFU MVA. Representative plots and mean  $\pm$  SEM from the combined data of two independent experiments with a total of 10 mice/group are shown. Statistical analysis Student's t test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; n.s., non-significant.

KLRG1<sup>low</sup> Ag-specific CD8<sup>+</sup> T cells, considered to have received CD4<sup>+</sup> T cell help, when CD169 interaction was blocked during vaccinia virus. This would imply that Ag transfer from CD169<sup>+</sup> macrophages to BATF3-dependent DCs is not only essential

Previously, CD169 was implicated in mediating binding of CD169<sup>+</sup> macrophages to lymphocytes, neutrophils, and innate lymphocytes (Crocker et al., 1995; van den Berg et al., 1992; Zhang et al., 2016). Here, we show strong CD169 binding to DCs, and we propose that this adhesive function promotes Ag transfer especially to BATF3-dependent CD8 $\alpha$ <sup>+</sup> cross-presenting DCs. This pathway will possibly be less relevant for those Ags and viruses that are likely to be taken up directly by DCs, such as soluble Ag, immune complexes, and VSV. DCs express multiple Fc $\gamma$  and complement receptors that enable immune complex uptake and cross-presentation (den Haan and Bevan, 2002; van Montfort et al., 2007). In addition, effector T cell priming after VSV infection has been previously shown not to require CD169<sup>+</sup> macrophages (Ciavarella et al., 1997; Honke et al., 2011; Iannacone et al., 2010). Interestingly, a recent study demonstrated that specifically memory CD8<sup>+</sup> T cell responses for VSV were dependent on XCR1<sup>+</sup> cross-presenting DCs and their interaction with CD169<sup>+</sup> macrophages (Habbedine et al., 2017). Collectively, these studies indicate that XCR1<sup>+</sup> BATF3-dependent CD8 $\alpha$ <sup>+</sup> cross-presenting DCs are differentially required for effector and memory CD8<sup>+</sup> T cell responses in different viral infections. Our results with the non-replicative vaccinia virus infection model suggest that effector and memory T cell responses that are dependent on cross-presenting DCs may be influenced by CD169-mediated transfer of Ag.

Presentation by BATF3-dependent DCs serves as a platform for CD4<sup>+</sup> T cell help that is necessary for the generation of long-term CD8<sup>+</sup> T cell memory responses and mediates generation of CD8<sup>+</sup> T cell resident memory responses (Eickhoff et al., 2015; Hor et al., 2015; Iborra et al., 2016). Ag transfer from CD169<sup>+</sup> macrophages to BATF3-dependent DCs would optimally facilitate the generation of these (resident) memory CD8<sup>+</sup> T cell responses. Indeed, we observed a decrease in

for the generation of effector CD8<sup>+</sup> T cell responses, but also enables CD4<sup>+</sup> T cell help and long-term memory T cell responses.

In conclusion, we demonstrate that CD169<sup>+</sup> macrophages interact with cross-presenting CD8 $\alpha$ <sup>+</sup> DCs in a CD169-dependent manner and thereby enable Ag transfer, DNGR-1 stimulation, and the cross-priming of CD8 T cell responses. We propose that this collaboration is optimized for Ag acquisition and Ag presentation to T cells, is physiologically relevant, and could also be exploited for vaccination purposes.

## EXPERIMENTAL PROCEDURES

### Preparation of Monoclonal Antibody-OVA Conjugates

Purified rat IgG2a Ab  $\alpha$ CD169 (MOMA-1),  $\alpha$ DEC205 (NLDC145), and isotype control (R7D4) were activated with SMCC (Pierce). Lipopolysaccharide (LPS)-free OVA (Seikagaku) was functionalized with SATA (Pierce). After removal of excess reagent and deacetylation of SATA, Abs were conjugated with 5 equivalents (molar ratios) of OVA. Unconjugated OVA was removed by gel filtration using a Superdex 200 column (Amersham).

### Mice

C57Bl6/J, BATF3-deficient, and OT-I mice were obtained from Charles River or the Jackson Laboratory. Sn-KI mice in which Trp2 and Arg97 from CD169 were changed to Gln and Ala, respectively, were generated at the University of Dundee, and bred at the animal facility of the VU University Medical Center (Klaas et al., 2012). *Clec9a<sup>gfp/gfp</sup>* mice were bred at the animal facility of the CNIC in Madrid, Spain (Sancho et al., 2009). All mice used in this study were matched for age (8–12 weeks) and sex and kept under specific pathogen-free conditions. Experiments were approved by the Animal Ethics Committees of our institutes.

### Immunizations

Mice were immunized intravenously (i.v.) with 1  $\mu$ g monoclonal antibody (mAb)-OVA in the presence or absence of 25  $\mu$ g poly(I:C) and 25  $\mu$ g  $\alpha$ CD40 Ab (clone 1C10). Wild-type mice were treated with CD169-blocking Ab (mix of 200  $\mu$ g SER-4 and 100  $\mu$ g 3D6) or isotype control Ab (300  $\mu$ g R7D4) at (day –1 or –2) and infected i.v. with  $2 \times 10^6$  plaque-forming unit (PFU) of

VSV or infected in the footpad with  $5 \times 10^5$  PFU MVA. MVA-GFP was kindly provided by Mariano Esteban (Di Pilato et al., 2013). VSV (Indiana strain, Mudd-Summers isolate) was originally obtained from D. Kolakofsky. Virus was propagated on BHK-21 hamster kidney fibroblasts at a MOI of 0.01.

### Flow Cytometry

Stainings were performed after blocking Fc receptors with clone 2.4G2 for 30 min at 4°C. For intracellular staining, cells were fixed in PBS containing 2% paraformaldehyde and stained in PBS with 0.5% BSA and 0.5% saponin. Cells were analyzed using a Cyan ADP flow cytometer (Beckman Coulter), Calibur (BD Bioscience), or Fortessa (BD Biosciences) and the FlowJo software package (Tree Star). For gating strategies, see Figure S7.

### Antibodies and Plant Lectins

The following antibodies were used from eBioscience: anti-B220 (clone RA3-6B2), anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-Ly-6G/Gr-1 (clone RG6-8C5), anti-CD4 (clone RM4-5), anti-CD11a (clone M17/4), anti-IFN $\gamma$  (clone xM61.2), anti-VCAM-1 (clone 429), Fixable Viability Dye eFluor 780 and from Biolegend: anti-CD8 $\alpha$  (clone 53-6.7), anti-VCAM-1 (clone 11-26c.2a). We produced and labeled the following antibodies in house: anti-MHC-II (clone M5/114), anti-CD169 (clones MOMA-1-488 or SER-4-488), isotype control (clone R7D4). Plant lectins were obtained from vector labs: Maackia Amurensis Lectin II (MALII) and Sambucus Nigra Lectin (SNA). Sytox blue nucleic acid stain (Invitrogen).

### Immunofluorescence

Tissue sections were cut at 5–6  $\mu$ m Mikrom HM 560 cryostat, fixed in 100% acetone, stained, and analyzed with a LEICA DM6000 or Leica TCS SP8 equipped with a pulsed white light laser and HyD hybrid detector equipped with temporal gating. LAS AF software was used for acquisition and ImageJ for image processing subsequent to data acquisition. Huygens professional deconvolution software was used for deconvolution.

### Histocytometry

Spleen tissue was cryosectioned at 16  $\mu$ m, and sections were rehydrated in 0.1 M Tris buffer for 30 min and blocked with blocking buffer (0.1 M Tris buffer, 0.02% Triton X-100, 1% normal mouse serum, anti-CD16/32 blocking Ab, 1% BSA) for 1 hr. Tissue sections were stained with directly labeled primary antibodies in blocking buffer for 16 hr at 4°C. After an extensive wash with 0.1 M Tris buffer, sections were mounted in Fluoromount-G (Thermo Fisher). Nuclei were counterstained with JOJO-1 Iodide (Thermo Fisher). Whole spleen cross-sections were acquired (voxel size  $y \times x = 240$  nm,  $z = 1$   $\mu$ m) on a Leica TCS SP8 confocal microscope with tunable pulsed white-light laser, 405 nm violet laser, and Leica HyD hybrid detectors. After acquisition, the tiled images were merged and compensated using the LAS X Merge and Channel Dye Separation module, respectively. Next, 3D stacked and compensated images were analyzed using histocytometry in Imaris 7.4 (Bitplane) and FlowJo as previously described (Gerner et al., 2012). In short, CD169 signal was used to segment CD169 $^{+}$  macrophages by surface rendering and splitting individual cells using the Surface Module. The segmentation of dendritic cells was obtained by first creating a masking channel by selecting for B220 $^{+}$ CD3 $^{+}$ MHCII $^{+}$ CD11c $^{+}$  voxels. The expression of CD4, CD8, MHCII, and CD11c on this masking channel was used as “true” DC expression of these markers. Next, a “dendritic cell” channel was created by combining CD11c $^{+}$  MHCII $^{+}$  voxels on the B220 $^{+}$ CD3 $^{+}$ MHCII $^{+}$ CD11c $^{+}$  mask. This channel was used to segment and split individual dendritic cells using the Imaris Surface Module (Bitplane). Next, individual surfaces were exported to FlowJo and used for gating and figure representation.

### Analysis of CD8 and CD4 T Cell Responses

Splenocytes from mice 7 days after i.v. immunization were re-stimulated *in vitro* for 5–6 hr with MHC class I restricted OVA<sub>257–264</sub> peptide (0.1  $\mu$ g/mL), MVA-derived B8R<sub>20–27</sub> peptide, or VSV antigen p8 peptide or p52 peptide including GolgiPlug or Brefeldin A or for 18 hr with MHC class II restricted OVA<sub>262–276</sub> peptide (100  $\mu$ g/mL) followed by 5 hr incubation with GolgiPlug (BD Biosciences). For tetramer stainings, splenocytes were incubated 45 min at 37°C with PE-labeled H2-K $^b$ :SIINFEKL tetramers (generated at LUMC, Leiden, the Netherlands) in the

presence of anti-CD8 Ab (clone 53-6.7). APC-labeled H-2K $^b$  B8R<sub>20–27</sub> tetramers were provided by the NIH Tetramer Facility at Emory University.

### Spleen Digestion

Spleens were cut and incubated in 1 mL per spleen of 2 WU/mL Liberase I (Roche Diagnostics GmbH, Mannheim, Germany) and 50  $\mu$ g/mL DNase I (Roche) in medium without fetal calf serum (FCS) and  $\beta$ -mercaptoethanol with continuous stirring at 37°C for 15 min. EDTA was added to a 10 mM final concentration, and the cell suspension was incubated for an additional 5 min at 4°C. Red blood cells were lysed with ammonium-chloride-potassium lysis buffer. Cells were washed once and undigested material was removed by a 70–100  $\mu$ m filter.

### Ex Vivo Ag Presentation Assays

Mice were injected i.v. with 1  $\mu$ g mAb-OVA plus 25  $\mu$ g  $\alpha$ CD40 and 25  $\mu$ g poly(I:C). After 16 hr, DCs from 5–15 spleens were purified with anti-CD11c MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and co-cultured ( $2.5 \times 10^5$ ) with purified OT-I T cells ( $10^5$ ) in triplicate in flat-bottomed 96-well plates. As a positive control, DCs were incubated with 1  $\mu$ g/mL MHC class I OVA<sub>257–264</sub> peptide for 1 hr and washed three times. After 48 hr, plates were pulsed with 1  $\mu$ Ci/well of [ $^3$ H]-thymidine. After an additional 16 hr, [ $^3$ H]-thymidine incorporation was measured on a Wallac-LKB Betaplate 1205 liquid scintillation counter. Data were summarized by the mean and SEM.

### In Vivo Ag Transfer Assays

Mice were immunized i.v. with 1  $\mu$ g mAb-Alexa488 or mAb-biotin in the presence or absence of 25  $\mu$ g poly(I:C) and 25  $\mu$ g  $\alpha$ CD40 Ab 1C10. Ten minutes or 2 hr after injection, mice were sacrificed and spleens were digested for fluorescence-activated cell sorting (FACS) analysis or frozen for immunofluorescence analysis.

### Imaging Flow Cytometry

After digestion, splenocytes from three mice were pooled, stained for surface markers, and fixed in 2% paraformaldehyde. Cells were analyzed using the ImageStreamX (Merck Millipore) imaging flow cytometer. Internalization scores were calculated as previously described (García-Vallejo et al., 2014). In short, a mask was designed based on the bright-field morphology image that was eroded until the membrane was left out of the mask. Alexa488 signal within this mask was defined as intracellular, while Alexa488 signal outside was defined as membrane-associated. The internalization score was calculated using the internalization feature provided in Ideas v6.0 software (Merck Millipore). Scores below 0 indicate that the majority of the signal is membrane-associated, while scores above 0 indicate that the majority of the signal is intracellular.

### Lectin and CD169 Binding Assays and Neuraminidase Treatment

Biotinylated plant lectins from Maackia amurensis Lectin-II (MAL-II) and Sambucus nigra (SNA) were incubated with DCs for 30 min at 37°C in TSM buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM CaCl<sub>2</sub>, and 2 mM MgCl<sub>2</sub>) with 0.5% BSA (Fluka Biochemika), followed by additional stainings at 4°C. For CD169-Fc binding assays, DCs were incubated with pre-complexed CD169-Fc or CD169-Fc-R97A (1–10  $\mu$ g/mL) with goat-anti-Human-Fc-Alexa488 (0.5  $\mu$ g/mL, Invitrogen) in TSM buffer with 1% BSA (Fluka Biochemika) for 45 min at room temperature or 1 hr on ice and analyzed by flow cytometry. For neuraminidase treatment cells were incubated in DMEM without FCS with 1:60 sialidase (Sigma) for 1 hr at 37°C and mixed gently every 15 min.

### Statistical Analysis

Statistical significance was tested using GraphPad Prism by performing a two-tailed student-t test, ANOVA with Bonferroni's correction, or a Kruskal-Wallis test with Bonferroni's correction (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; n.s., non-significant). N represents the number of animals used in the experiment.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.01.021>.



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## AUTHOR CONTRIBUTIONS

Experimental design by D.v.D., H.V., M.R.B., D.S., K.S.L., L.B.C., P.R.C., and J.M.M.d.H. Experiments were conducted by D.v.D., H.V., S.I., E.G.F.B., L.H., K.O., S.T.T.S., K.S.L., and M.R.B. H.K. and K.L.F. synthesized conjugates and tetramers. Data analysis by D.v.D., H.V., S.T.T.S., S.I., K.S.L., M.R.B., and J.J.G.-V. The manuscript written by J.M.M.d.H. and D.v.D. and edited by all authors.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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